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SPATIAL CONTROL OF POSTSYNAPTIC PROTEINS

– A role in brain plasticity

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ABSTRACT

Neurons communicate via synapses; the strength of each synapse is defined both pre and postsynaptically. Presynaptically, the strength of the synapse is defined by which neurotransmitter is being released and how much. On the postsynaptic membrane a corresponding receptor will receive the transmitter. Receptor abundance and availability determines the strength of the synaptic connection.

Protein function is as tightly linked to structure as it is to location. Due to the fluidic nature of the plasma membrane, any membrane protein will be highly mobile unless it is being anchored or confined within a compartment by an intracellular protein or cytoskeletal complex. High mobility facilitates interactions between proteins and ensures proper localization through free energy minimization without the need of directed transport. The dynamic regulation of protein mobility is fundamental in defining the function of the protein. The overall abundance and availability of postsynaptic proteins are dependent on many processes such as exocytosis/endocytosis, activation/inactivation, and lateral diffusion.

The aim of this thesis was to study how postsynaptic proteins can be regulated in the dendritic membrane by availability and mobility.

Dopamine is an important modulatory neurotransmitter that is involved in cognition, memory, motoric functions and reward-mechanisms.

Calcyon is an accessory protein that has been suggested to modulate dopamine receptor signaling. We show that calcyon is a neuron-specific vesicular protein with a high intracellular mobility. Furthermore we show that calcyon forms vesicular clusters located just beneath the plasma membrane. We propose a role for calcyon in the trafficking of proteins that are important for synaptic plasticity, to and from the dendritic plasma membrane.

Dopamine receptors are divided into two groups, the D_1-like and D_2-like group, each with distinct downstream signaling pathways. We show that the two isoforms of the D_1-like group, the D_1 and D_5 receptors have distinctly different subcellular localization in striatal neurons and interact differently with the NMDA receptors. We propose that the two isoforms, due to differences in localization and interactions with other receptors, have distinct roles in neuronal dopaminergic signaling.

Most G-protein coupled receptors are transported to the plasma membrane of the soma and are then transported via lateral diffusion to the site of action. We studied several GPCRs, involved in mood regulation and behavior, to elucidate whether GPCRs share a common mode of transport. We show that the 5-HT_{1B} receptor, in contrast to other GPCRs, is transported in vesicles in the lumen of the dendrites. We show that the vesicles release the receptors to the membrane close to inhibitory synapses, followed by subsequent lateral diffusion and confinement in inhibitory as well as excitatory synapses. We propose that this special mode of transport serves as an additional mode of regulation, which enables fine-tuning of serotonergic signaling.

The Na,K-ATPase is an essential ion transporting protein that is found in all cells where it is responsible for the generation of the plasma membrane ion gradient that is the driving force for many important cellular processes. Different isoforms of the catalytic, ion-pumping, α subunit are expressed in different cell types. We show that the neuron-specific α3 isoform is responsible for the sodium clearance in dendrites following synaptic signaling, which is essential for proper neuronal function. Furthermore we show that the α3 subunit is highly mobile in the postsynaptic membrane and that it is confined in excitatory synapses. We show that mobility is modulated by neuronal activity; excitatory stimulation results in an increased mobility in both the synaptic as well as the extrasynaptic region.

LIST OF PUBLICATIONS

I. **Markus Kruusmägi**, Sergey Zelenin, Hjalmar Brismar, Lena Scott.

Intracellular dynamics of calcyon, a neuron-specific vesicular protein. *NeuroReport*, 18(15), 1547-1551, 2007.

II. **Markus Kruusmägi**, Sandeep Kumar, Sergey Zelenin, Hjalmar Brismar, Anita Aperia, Lena Scott.

Functional differences between D1 and D5 revealed by high resolution imaging on live neurons. *Neuroscience* 164(2), 463-469, 2009.

III. Thomas Liebmann, **Markus Kruusmägi**, Nermin Sourial-Bassillious, Alexander Bondar, Per Svenningsson, Marc Flajolet, Paul Greengard, Lena Scott, Hjalmar Brismar, Anita Aperia.

A non-canonical postsynaptic transport route for a GPCR belonging to the serotonin receptor family. *Accepted in Journal of Neuroscience, Oct. 2012.*

IV. Guillaume Azarias*, **Markus Kruusmägi***, Siobhan Connor*, Evgeny E. Akkuratov, David Lyons, Hjalmar Brismar, Christian Broberger, Anita Aperia.

A specific and essential role for Na,K-ATPase α3 in neurons co-expressing α1 and α3. *Manuscript.*

* Equal contribution.

PUBLICATIONS NOT INCLUDED IN THIS THESIS

Juan Li*, Georgiy Khodus*, **Markus Kruusmägi**, Padideh Kamali-Zare, Xiao-Li Liu,
Ann-Christine Eklöf, Sergey Zelenin, Hjalmar Brismar, Anita Aperia.

Ouabain protects against adverse developmental programming of the kidney.
Nature Communications, 1:42, 2010.

* Equal contribution.

CONTENTS

LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
ATP	Adenosine-5'-triphosphate
cAMP	Cyclic adenosine monophosphate
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CME	Clathrin-mediated endocytosis
DNA	Deoxyribonucleic acid
EPSP	Excitatory Postsynaptic Potential
FRAP	Fluorescence Recovery After Photobleaching
GABA	γ-aminobutyric acid
GFP	Green Fluorescent protein
GPCR	G-protein coupled receptor
I_p	Na,K-ATPase specific current
IP_3	Inositol trisphosphate
LTD	Long-term depression
LTP	Long-term potentiation
NCX	Sodium/calcium exchanger
NKA	Na,K-ATPase
NMDA	N-methyl-D-aspartic acid
PKA	Protein kinase A
PKC	Protein kinase C
PSD	Postsynaptic Density
QD	Quantum Dot
SEP	Super Ecliptic pHluorin
SPT	Single particle tracking
VGCC	Voltage gated calcium channels
VGPC	Voltage gated potassium channels
VGSC	Voltage gated sodium channels

1 BACKGROUND

1.1 Neurons

Neurons are the smallest components of the mammalian brain, the most complex object known to man. There are many different types of neurons, with different sizes and shapes. A typical neuron consist of a cell body, soma, one or several branches, dendrites, that receive signals from other neurons and the axon which is the output structure that sends information to other neurons (Figure 1.1). The principal function of the neuron is to receive information, process the information (synaptic integration) and to send an output signal to receiving neurons. Each neuron makes thousands of connections with other neurons. Considering that the estimated number of neurons in the human brain is 100 billion (Azevedo et al., 2009), this yields an enormous amount of connections.

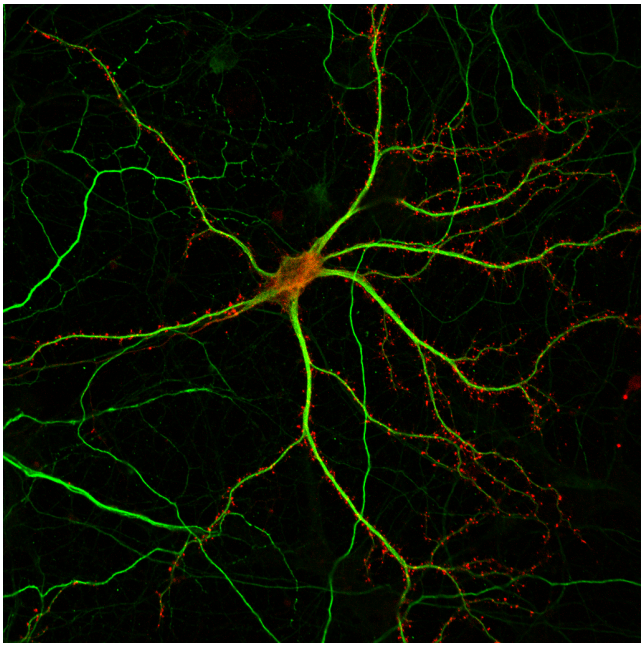

Figure 1.1: Neuron.

1.1.1 Membrane potential

The plasma membrane of a cell is a lipid bilayer acting as a barrier between the inside of the cell, the cytosol, and the outside, the extracellular space. An ion gradient exists over the membrane formed by ion differences between the inside and the outside of the cell. The Na,K-ATPase is an electrogenic pump that is responsible for generating the ion gradient by pumping three sodium ions out and two potassium ions into the cell for each cycle at the expense of one

molecule of ATP (Kaplan, 2002; Skou and Esmann, 1992). The inside of the plasma membrane is highly negative due to negatively charged proteins that are incorporated in the membrane. The charge difference and the ion gradient over the membrane generate an electrochemical force that drives many important processes in mammalian cells. Specific ion channels in the plasma membrane let certain species of ions pass through. The membrane potential is mainly built up by the chemical gradient driving positively charged potassium ions through channels into the cell. The potential is in the range of -40 to -80 mV relative to the outside of the cell. The resting potential is the steady-state situation when the electrical and chemical forces are at equilibrium. The membrane potential is important for all cells but is essential for neurons. Excitable cells, such as neurons and muscle cells, have a large collection of ion channels and other ion regulating units that control the flow of ions across the lipid bilayer thus modulating the membrane potential of the cell.

Neuronal activity alters the membrane potential, either making it less negative or more negative, which is called depolarization or hyperpolarization, respectively. Neurons can receive both excitatory and inhibitory signals. An excitatory signal will depolarize the neuron, pushing it toward a more excited state while an inhibitory signal will do the opposite: hyperpolarize the cell, lowering the excited state. If the sum of these signals results in a depolarization that is above a certain threshold, the cell will trigger an action potential that is generated at the axon hillock and is conducted through the axon out to the axon terminal which subsequently will trigger a signal to the receiving cell.

1.2 The Synapse

A connection between two neurons is called a synapse (Bennett, 1999). The neuron that is sending information is defined as presynaptic while the receiving neuron is designated postsynaptic. Synapses can be either electrical or chemical. The most common type is the chemical synapse which is slower than the electrical synapse but which instead is more diverse in types of propagated signals and which has the capability of amplifying the input signal (Hormuzdi et al., 2004). The focus of this thesis is to study the regulation of postsynaptic membrane proteins located at, or in the vicinity of, chemical synapses.

1.2.1 Chemical Synapse

A chemical synapse is divided into two parts: the presynaptic terminal or bouton, which is formed on the axon of the presynaptic neuron; and the postsynaptic membrane, typically located on a dendrite of the postsynaptic neuron. The bouton holds neurotransmitter-containing vesicles positioned

directly beneath the presynaptic membrane. When an action potential is transmitted from the presynaptic cell through the axon to the bouton, the resulting depolarization of the membrane will trigger an influx of calcium through voltage-gated calcium channels. The increased intracellular calcium will trigger docking and fusion of neurotransmitter-containing vesicles with the plasma membrane and the subsequent release of neurotransmitter into the space between the pre- and postsynapse, the synaptic cleft. The neurotransmitters are recognized by specific receptors, located in the postsynaptic membrane, which will in turn relay the information to the receiving postsynaptic cell. Depending on the type of neurotransmitter and the type of receptor, the transmitted signal will be excitatory, inhibitory or modulatory i.e. the receptor might modify the function of other receptors. The type of neurotransmitter depends on the type of neuron; neurons that signal via the excitatory neurotransmitter glutamate are called glutamatergic neurons, while neurons that transmit the inhibitory compound GABA are called GABAergic. Some nerve terminals contain more than one neurotransmitter. The strength of the synaptic signal depends on the number of neurotransmitter containing vesicles, the amount of neurotransmitter in each vesicle and on the number of matching receptors in the receiving postsynaptic membrane. Many pharmaceutical drugs work by modifying the abundance of neurotransmitter in the synaptic cleft. One example is the selective serotonin reuptake inhibitors (SSRIs) that are used in the treatment of depression and that act by blocking the reuptake of the neurotransmitter serotonin, resulting in prolonged activation of the serotonin receptors.

1.3 Neurotransmitters

Neurotransmitters are small endogenous compounds that act as chemical signals between cells. Some are modulatory in nature and control mood and behavior such as the monoamines (e.g. 5-HT or serotonin) and the catecholamines (dopamine, adrenaline, noradrenaline). Others relay motoric or sensory signals such as acetylcholine. The main excitatory neurotransmitter is the amino acid glutamate, which is responsible for the majority of excitatory neurotransmission. The principal inhibitory neurotransmitter is gamma-aminobutyric acid (GABA).

1.3.1 Dopamine

Dopamine is the most abundant catecholamine neurotransmitter in the brain. It is synthesized in the midbrain by the neurons in the ventral tegmental area (VTA) and substantia nigra (SN). The dopaminergic neurons in VTA and SN

project to several brain areas and can be grouped into four major pathways. The nigrostriatal pathway, connects the SN with the striatum, this pathway is an important part of the basal ganglia and is primarily involved in motor control, cognitive functions and affection. The nigrostriatal pathway is strongly implicated in Parkinsonism. The mesocortical pathway connects VTA with the frontal cortex; this pathway is involved in normal cognitive function. The neurons in the mesolimbic pathway project from the VTA trough the nucleus accumbens to several areas of the limbic system such as hippocampus and amygdala. The mesolimbic pathway is responsible for reward mechanisms (Robbins and Everitt, 1996) and is the primary focus in addiction (Kalivas et al., 1993). The mesocortical and mesolimbic pathway are strongly implicated in the negative and positive symptoms of schizophrenia, respectively. The fourth dopaminergic pathway is the tuberoinfundibular pathway, which originates in the hypothalamic arcuate nucleus and projects to the median eminence, where the dopamine is released into the pituitary portal blood. Dopamine acts not only in the nervous system, it has also an important role in regulating blood pressure and salt secretion in the kidneys (Aperia, 2000).

1.3.2 5-HT, Serotonin

5-hydroxytryptamine (5-HT) or serotonin is a monoamine neurotransmitter synthesized from the essential amino acid tryptophan. There are several serotonergic pathways in the brain, all projecting from the seven groups of neurons called the raphe nuclei that are localized in the brain stem. The lower raphe nuclei (more caudal) project to the medulla and the spinal cord, while the upper nuclei (more rostral) project to the frontal cortex, thalamus, and areas of the limbic system such as neocortex and hippocampus. The serotonergic pathways are important for a number of functions such as memory processing and regulation of mood, cognitive functions and sleep. Many drugs are targeted to the serotonin system; antidepressants are often 5-HT reuptake inhibitors (SSRI) while psychedelic drugs such as LSD are agonists of 5-HT receptors.

1.4 G-protein coupled receptors

The G-protein coupled receptor (GPCR) protein family is a large group of receptors involved in many diverse signaling processes in mammalian cells including: vision via photo-receptors (rhodopsins) in the retina; movement and behavior via neurotransmitter receptors in the brain and in the autonomic nervous system regulating blood pressure and heart rate. Because of the diverse and essential role of GPCRs in the body, receptor dysfunction is

commonly found in many diseases and disorders, making GPCRs important pharmacological targets. (Rosenbaum et al., 2009).

1.4.1 Dopamine Receptors

Dopamine acts on dopamine receptors, a family of GPCRs that are divided into two groups based on their principal mode of signaling: the D_1-like family (D_1 and D_5), and the D_2-like family (D_2, D_3 and D_4) (Missale et al., 1998). The D_1-like family stimulates adenlylate cyclase (AC) via activation of $G_{\alpha S}$, triggering an increase in the production of the second messenger cyclic adenosine monophosphate (cAMP). Activation of a receptor that belongs to the D_2-like family on the other hand, activates $G_{\alpha i}$, which inhibits adenylate cyclase, causing a decrease in the production of cAMP. The downstream effect of the dopamine signaling will depend on the predominant receptor isoform in the postsynaptic membrane of the responding cell. The dopamine receptor isoforms are expressed in different part of the brain: high levels of the D_1 and D_2 isoforms can be found in striatum where cells are expressing either of the two isoforms (Cepeda et al., 2008).

Dopamine receptors are also found in prefrontal cortex (Seamans and Yang, 2004), where D_1 activation enhances and D_2 activation reduces glutamatergic transmission (Tseng and O'Donnell, 2004). It has previously been shown that dopaminergic afferents form symmetric synapses on spines of pyramidal neurons. These spines have an additional asymmetric excitatory synapse. It has been proposed that the dopaminergic synapse modulate the excitatory signal directly on the spine (Freund et al., 1984; Goldman-Rakic et al., 1989). D1-like receptors have also been shown to potentiate NMDA receptors via a PKC dependent mechanism in the nucleus accumbens (Chergui and Lacey, 1999).

The two isoforms of the D_1-like family, D_1 and D_5, are highly homologous; the transmembrane regions are almost identical, they differ mainly in their respective carboxy-termini. Agonist and antagonist affinities are similar for D_1 and D_5. D_5, however, have a ten-fold higher affinity for dopamine than D_1.

Dopamine dysregulation is linked to severe movement and psychiatric disorders such as Parkinsonism (Fearnley and Lees, 1991), Huntington's disease (Cepeda et al., 2007) and schizophrenia (Sawa and Snyder, 2002). These conditions are treated with pharmacological compounds that are targeting the dopamine system usually by strongly inhibiting one set of receptors. However,

many of them are often associated with adverse side effects. A better understanding of the differences in localization and spatial regulation of the different dopamine receptors is necessary in order to develop better drugs.

1.4.2 5-HT Receptors

The 5-HT receptors constitute a large group of receptors divided into seven families (5-HT_1 - 5-HT_7) based on structure and downstream signaling (Hannon and Hoyer, 2008).

Family	G-protein	Signal	Receptors
5-HT_1	$G_{\alpha i/o}$	cAMP ↓	5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-ht_{1E}, 5-HT_{1F}
5-HT_2	$G_{\alpha q/11}$	IP_3 DAG ↑	5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}
5-HT_3	Cation channel	Na^+ $Ca2^+$↑, K^+↓	5-HT_3
5-HT_4	$G_{\alpha s}$	cAMP ↑	5-HT_4
5-HT_5	$G_{\alpha i/o}$	cAMP ↓	5-HT_{5A}, 5-HT_{5B}
5-HT_6	$G_{\alpha s}$	cAMP ↑	5-HT_6
5-HT_7	$G_{\alpha s}$	cAMP ↑	5-HT_7

Table 1.1: 5-HT receptors, name and function. From (Hannon and Hoyer, 2008).

5-HT receptors are involved in the control of diverse brain functions such as mood regulation, sleep, memory and learning.
In paper III we study the 5-HT_{1B} receptor, which in mouse knockout studies has been implicated in the pathophysiology of addiction, aggression, depression, anxiety and sleep disorders (Gingrich and Hen, 2001; Moret and Briley, 2000; Saudou et al., 1994).

1.5 Accessory proteins

1.5.1 Calcyon

Calcyon is a single transmembrane domain protein that is predominately expressed in neurons. Calcyon was discovered when screening for proteins that interacts with the c-terminal domain of the D_1 (Lezcano et al., 2000). Its function was initially thought to alter the signaling pathway of the dopamine receptors, enabling the Gq pathway rather than the $G_{\alpha s}$ pathway. The name calcyon come from “Calcium on” and it was described that in HEK293 cells expressing both D_1 and calcyon, D_1 activation could trigger an increase in intracellular calcium (Lezcano et al., 2000). Later studies have shown that the initial report of a direct interaction between D_1 and calcyon was not correct

(Lezcano et al., 2006). The calcium stimulating effects of calcyon on D_1 could not be reproduced. Other studies have shown that D_1 could stimulate the pathway via $G_{\alpha q}$ rather than $G_{\alpha s}$ (Rashid et al., 2007; Undie and Friedman, 1990) but the role of calcyon in this process could not be verified.
However, continued investigations on the protein have shown that calcyon is a vesicular protein that is involved in clathrin mediated endocytosis (CME) (Jung and Haucke, 2007; Xiao et al., 2006) which is important in synaptic plasticity processes such as LTP and LTD (Park et al., 2004; Sheng and Kim, 2002).

1.6 Ion translocators (Channels/Pumps)

1.6.1 Glutamate Receptors

Glutamate is the main excitatory neurotransmitter in the brain. Glutamate activates both ionotropic and metabotropic receptors. The ionotropic receptors are named after the synthetic ligands that activate them: α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), N-methyl-D-aspartate (NMDA) and Kainic acid (Table 1.2).

Type	Subunits	Function
NMDA	NR1, NR2	Ionotropic
AMPA	GluR1-4	Ionotropic
Kainate	GluR5-7, KA1, KA2	Ionotropic
mGluR	mGluR1-8	Metabotropic

Table 1.2: Glutamate receptors.

The AMPA receptors (AMPA-R) are the main excitatory receptor in the brain. The abundance of AMPA-R in a synapse defines its strength. AMPA receptors form tetramers, composed of the four subunit isoforms, GluR1-4. The AMPA-R channel is permeable to Na^+, K^+. AMPA receptors are also permeable to Ca^{2+}, unless they contain the GluR2 subunit. The NMDA receptors are formed as a heterotetramer with at least one of the NR1 subunits and one or more of the NR2 subunits. The NMDA receptor, like the AMPA-R, is a non-selective cation channel that allows the flux of both Na^+ and Ca^{2+} into the cell and K^+ out of the cell.
The NMDA-R has a higher conductance than AMPA-R but is blocked by extracellular Mg^{2+} when the cell is at a resting state. Membrane depolarization is required to unblock the channel. The AMPA-R is responsible for the early phase and the NMDA-R is responsible for the late phase of the excitatory postsynaptic potential (EPSP).

1.6.2 Na,K-ATPase

The Na,K-ATPase is a plasma membrane protein that is expressed in virtually all cells (Blanco and Mercer, 1998; Kaplan, 2002; Skou and Esmann, 1992). By pumping three sodium ions out of the cell in exchange for two potassium ions at the expense of one molecule of ATP (**Figure 1.2**) the pump creates an ion gradient across the cell membrane that is driving many important processes in the cell. This chemical gradient is fundamental for the normal function of all cells and especially important for excitable cells such as neurons and cardiomyocytes.

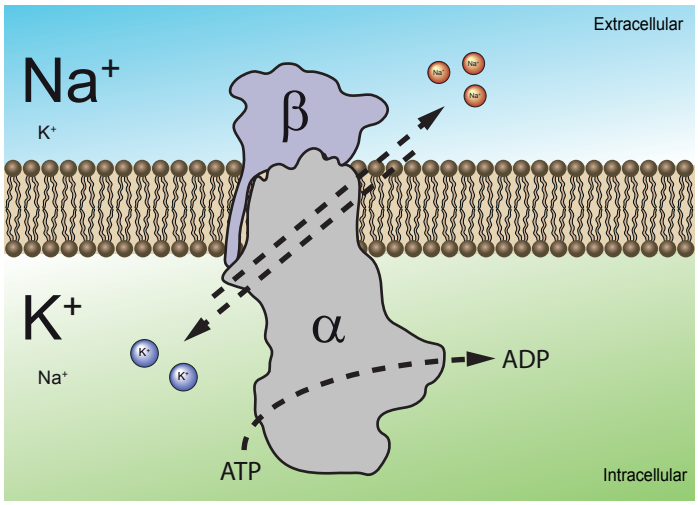

Figure 1.2: The Na,K-ATP. Three sodium ions are pumped out of the cell while two potassium ions are pumped in, at the expense of one molecule of ATP.

The Na,K-ATPase is a heterotrimeric complex constituting three subunits, alpha (α), beta (β), and gamma (γ). The α subunit is the catalytic, ion-pumping subunit, while the β subunit is responsible for translocation of the mature protein to the plasma membrane. The γ subunit is mostly described to have a regulatory role. The β subunit has also been shown to have a modulatory role of the overall pump activity. There are four different isoforms of the α subunit. α1 is the ubiquitous isoform that can be found in all cell types. α2 is expressed in the heart, in muscle cells and in astrocytes. The α3 isoform is almost exclusively expressed in neurons and the α4 isoform is expressed in testis.

The isoforms differ in terms of substrate affinity to sodium, potassium and ATP. Pump kinetics and sensitivity to ouabain also differ between isoforms. Some of these properties are summarized in table 1.3.

Isoform	Expression	$K_{0.5}$ $[Na^+]_o$ mM	$K_{0.5}$ $[K^+]_i$ mM	K_m $[ATP]_i$ mM	K_i Ouabain
α1	All cells	16.4	1.9	0.46	43 µM
α2	Heart, Brain	12.4	3.6	0.11	170 nM
α3	Neurons	27.9	5.3	0.09	31 nM
α4	Testis	13.5	5.9	0.19	6.4 nM

Table 1.3: Properties of the rat α-isoforms expressed in Sf-9 insect cells. From (Blanco, 2005).

The maximum turnover rate of the Na,K-ATPase at 37 °C is in the range of 150 – 200 cycles per second (Lupfert et al., 2001; Martin and Sachs, 1999).

In granular cells in the dentate gyrus of hippocampus it has been estimated that the Na,K-ATPase density needed to handle the basal activity in axons is approximately 50 pumps/µm^2. During bursts of high activity the pump density needs to be as high as 500/µm^2 (Alle et al., 2009).

The Na,K-ATPase has other functions in the cell besides pumping ions. Low doses of ouabain have been shown to trigger intracellular calcium oscillations via the Na,K-ATPase (Aizman et al., 2001). These oscillations activate NF-κB translocation and have an anti-apoptotic, tissue-protective effect. This has been described in the embryonic rat kidney, where low doses of ouabain protect the kidney from the negative effects associated with malnourishment (Khodus et al., 2011). Src kinase activation is another well described downstream effect of ouabain binding to Na,K-ATPase (Xie and Askari, 2002).

1.7 Spines

Dendritic spines are protrusions in the plasma membrane forming morphological specializations that separate parts of the plasma membrane from the rest of the dendrite. The functional role of the spine is a matter of debate (Shepherd, 1996), but there is a general consensus that spines forms a contact point for synaptic connections. One example of this is spines on medium spiny neurons (MSN) in striatum which form two type of connections, the excitatory glutamatergic synapse on the head of the spine and the modulatory dopaminergic synapse on the stem of the spine (Freund et al., 1984). Spines act also as isolated biochemical compartments packed with enzymes and signaling molecules involved in many different important functions, ranging from signal transduction to memory formation. Some spines even include the complete protein synthesis apparatus (Steward and Schuman, 2001; Sutton and Schuman, 2005). The spine is the active site of many proteins discussed in this thesis. The glutamate receptors have a prominent role in the

postsynaptic density (PSD) where they define synaptic strength. The number of AMPA receptors in hippocampal CA1 spines has been estimated to be approximately 150 per spine (Matsuzaki et al., 2001). The D_1 receptors and many other GPCRs are also found in spines where they are involved in the modulation of excitatory transmission.

1.8 Plasticity

Synaptic plasticity is the change in signal transmission between neurons, and is a combination of altered connectivity, i.e. a gain or loss of synaptic connections, and changes in synaptic signaling efficacy. The most studied form of synaptic plasticity relates to excitatory glutamate transmission where synaptic strength almost exclusively is a function of postsynaptic AMPA receptor abundance. The timescale of synaptic plasticity can be in the range of seconds to days. Two well-studied processes are long-term potentiation and long-term depression, LTP and LTD respectively. LTP was first described by Timothy Bliss and Terje Lømo in 1973. They studied granule cell neurons in the dentate gyrus of hippocampus, and found that brief trains of high frequency stimulation of the perforant path fibers, axons that innervate dendrites of the granular cells, resulted in a persistent potentiation of signaling that lasted for 30 minutes to 10 hours (Bliss and Lomo, 1973). This finding triggered a series of studies, and we now know that this form of long-term potentiation is dependent on the activation of NMDA receptors and calcium increases in spines (Bredt and Nicoll, 2003). LTP and LTD are associated with changes in AMPA receptor abundance in the spine head. LTP induction triggers a recruitment of GluR1 subunits into synapses (Hayashi et al., 2000) while LTD has been shown to cause a downregulation of AMPA receptors without altering the abundance of NMDA receptors in the postsynaptic density (Carroll et al., 1999). The spine head has been shown to increase or decrease in size as a consequence of LTP or LTD, respectively (Bosch and Hayashi, 2012). Spine head enlargement has been shown to be dependent on actin-polymerization and activation of Ca^{2+}/calmodulin-protein kinase II (CaMKII) (Matsuzaki et al., 2004). This change is likely induced in order to accommodate the changes in AMPA receptor quantity.

While AMPA receptors define synaptic strength, NMDA receptors are necessary in order to trigger changes in AMPA receptor abundance. The modulatory neurotransmitter receptors, GPCRs such as the D_1 receptor, add an additional layer of complex modulation to synaptic signaling. GPCRs have been shown to modify the abundance and potency of AMPA and NMDA receptors via phosphorylation processes (Greengard, 2001; Greengard et al., 1999).

2 AIMS

The overall aim of this thesis has been to elucidate the spatial regulation of selected postsynaptic proteins in the dendritic membrane and the potential role of this regulation in brain plasticity.

The specific aims of my studies were:

- To study the functional role of the brain-specific membrane protein Calcyon in neurons.

- To explore the difference between D_1 and D_5 receptors in terms of mobility, localization and response to glutamatergic activity.

- To study how GPCRs are transported to the postsynaptic site of action.

- To investigate the role of the brain-specific Na,K-ATPase isoform α3 in neurons and to elucidate to which extent the lateral diffusion of Na,K-ATPase is regulated by synaptic activity.

3 METHODOLOGICAL CONSIDERATIONS

3.1 The Green Fluorescent Protein

Optical microscopy has been around for centuries while fluorescence microcopy is a relatively modern invention. The real breakthrough in fluorescence microscopy for use in biology came with the discovery and development of the green fluorescent protein, GFP.

The GFP was discovered in the jellyfish Aequorea Victoria by Osamu Shimomura in 1962, but it took three decades before the primary structure of GFP was solved by Prasher et al. in 1992 (Prasher et al., 1992), and finally in 1994 it was used for the first time as a marker for gene expression (Chalfie et al., 1994). In 2008 Osamu Shimomura, Martin Chalfie and Roger Tsien were awarded the Nobel Prize in chemistry for the discovery of the GFP.

I have used GFP in one form or another in most of my projects. The many different variants of GFP constitute a formidable toolkit for use in biological research. We have used GFP, and GFP mutants with different colors, to visualize the intracellular expression and mobility of various proteins. We have used a pH-sensitive variant of GFP (Miesenbock et al., 1998) to study recruitment of receptor-containing vesicles to the plasma membrane and photoactivatable GFP (Patterson and Lippincott-Schwartz, 2002) to study the transport of nascent proteins from soma out to dendrites in neurons. We have aslo used GFP as ahighly specific epitope for antibody labeling in single particle tracking experiments.

A special type of genetically encoded calcium indicators (GECIs) are basesd on GFP, such as GCaMP (Nakai et al., 2001). The combination of GECIs and transgenic mice is particularly intriguing (Chen et al., 2012).

3.2 Intracellular ion measurements

The use of ion-sensitive dyes in microscopy has enabled researchers to investigate important processes inside cells. A milestone in the measurement of ions came in 1985 with the development of the ratiometric calcium indicator Fura-2 (Grynkiewicz et al., 1985). A few years later the same group developed the ratiometric sodium indicator sodium-binding benzofuran isophthalate (SBFI) (Harootunian et al., 1989).

Both Fura-2 and SBFI require ultra violet (UV) excitation (340 and 380 nm), which can be problematic in some circumstances. Because UV-lasers require special optics, it is not possible to use classical confocal microscopy. However, SBFI together with two-photon excitation was successfully utilized in several

elegant studies on sodium dynamics in neurons (Rose and Konnerth, 2001; Rose et al., 1999).

Since the introduction of SBFI, several indicators have been developed, all with different properties, summarized in **table 3.1**.

In our studies we have used the relatively new indicator, Asante Natrium Green (ANG). The benefits of ANG are the large signal-to-noise ratio, and a large response to change in sodium concentration ($\Delta F/F_0$), which has enabled us to measure sodium in distal dendrites (Paper IV). The disadvantage of ANG is a high K_d for sodium (80 mM), which makes it less sensitive in low sodium concentrations.

Indicator	K_d Na^+ (mM)	Excitation	Notes
SBFI	~10	340/380 nm	UV excitation
Sodium Green	~21	488 nm	488 excitation, low $\Delta F/F_0$
CoroNa Green	~80	488 nm	Leaks out of the cell
Asante Natrium Green-1	85	488-517 nm	Large $\Delta F/F_0$
Asante Natrium Green-2	20	488-517 nm	Large $\Delta F/F_0$

Table 3.1: Sodium indicators.

3.3 Protein mobility

Two of the most common methods to study membrane protein mobility are fluorescence recovery after photobleaching (FRAP) and single particle tracking (SPT). The next two sections will describe those methods, followed by a comparison of the two techniques.

3.3.1 Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) is a technique used to study the diffusion of molecules in a system. In biology it is commonly used to study the diffusion of fluorescently tagged membrane proteins. It was first described in a study of the lateral motion of acetylcholine receptors in membranes of muscle fibers (Axelrod et al., 1976). In FRAP, fluorescent molecules are irreversible bleached using high intensity light, e.g. a focused laser beam, in a well-defined region. The recovery rate of fluorescence into the bleached region is a measure of the diffusion of the fluorescent molecules. An example of FRAP can be viewed in **figure 3.1**, where the diffusion of GFP-tagged Na,K-ATPase into the spine of a hippocampal neuron is measured. The recovery halftime is the time it takes to reach half-maximal recovery. The mobile pool is the proportion of fluorescence at maximum recovery to the initial fluorescence

(**Figure 3.1B**). The mobile pool will be 100%, i.e. fluorescence returns to initial level, if all fluorescent molecules are completely free to move and are not obstructed by barriers or other molecules in the bleached region. FRAP is traditionally visualized in intensity vs. time plots. Another visualization method is the kymograph, which shows additional information, such as flow direction and sub-regional variations (**Figure 3.1D and E**). Note that in the kymograph in figure 3.1E, it is apparent that the fluorescent molecules are transported from the dendrite into the head of the spine. In paper III we used this technique to observe mobility of dendritic 5-HT_{1B} clusters.

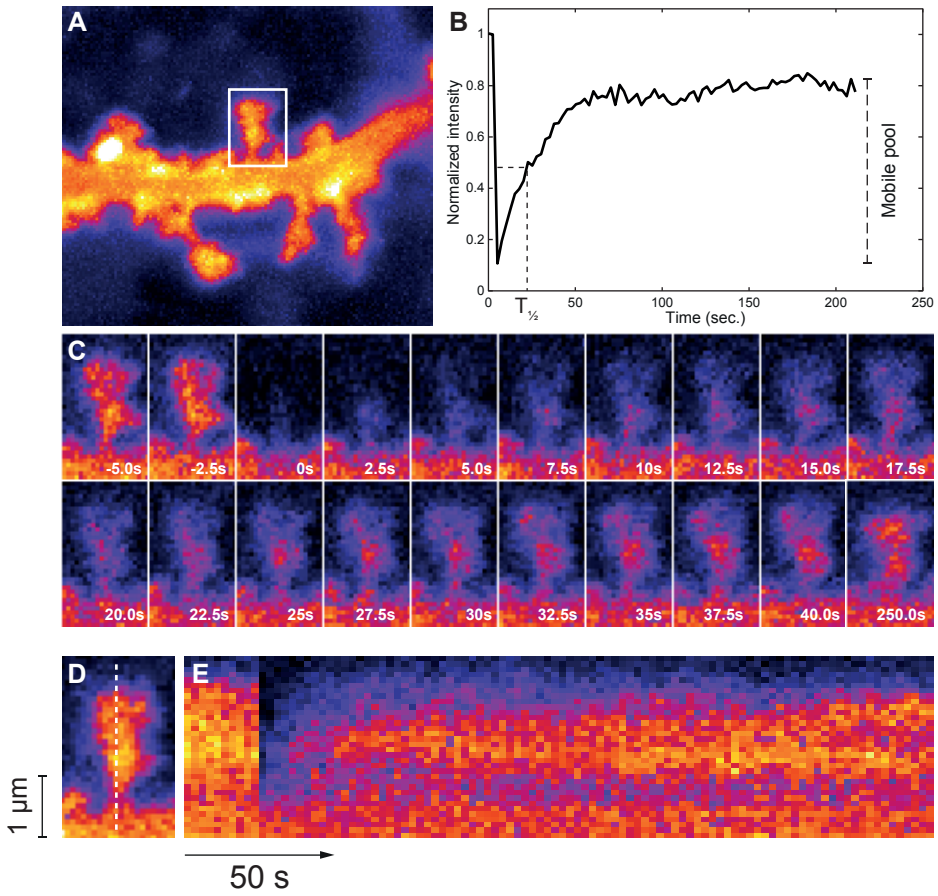

Figure 3.1: Example of FRAP recording on dendritic spine. A) Dendritic branch with spines expressing SEP-NKA-α3. White box defines the bleach region. B) Recovery plot recorded from the region defined by the white box in A. $T_{½}$ is the recovery halftime, defined as time for 50% recovery. Mobile pool is the proportion of final recovery to initial, pre-bleach, intensity. C) Time points from the time-lapse recording showing the diffusion of α3 molecules from the dendrite into the head of the spine. Time point at 0s is directly after the bleach step. D,E) Kymograph visualizing the recovery. Kymographs are two-dimensional plots with time vs. spatial dimension. The dotted line in D defines the projection line used for the kymograph in E.

3.3.2 Single Particle Tracking

Single particle tracking (SPT) is a technique used to monitor the mobility of a single object over time. Depending on the particular question, the object can be a molecule, protein, or protein complex. The technique is distinct from other mobility-measuring techniques like FRAP where the population mobility or diffusion of many particles at the same time is measured.

The first SPT study on cell membranes was of low density lipoprotein-receptor complex diffusion using fluorescent diI-LDL (Barak and Webb, 1982). Later a technique used in the study of microtubule dependent transport using video microscopy on 20-40 nm gold beads was dubbed nanovid microscopy (De Brabander et al., 1985). Since then many different techniques have been developed to track proteins and complexes. Latex beads and differential interference contrast microscopy have been employed to study the lateral movement of AMPA receptors (Borgdorff and Choquet, 2002), but due to the large size of the latex beads, the complex was blocked from entering the synaptic cleft. Organic dyes were later used to enable the tracking of the lateral movement of AMPA-R in the synapse (Tardin et al., 2003). Organic dye molecules, like Cy3, are very small, which is an advantage in SPT, but they can only be tracked for a couple of seconds due to photobleaching. Quantum dots (QD) are semiconductor crystals that are extremely photostable, they have a relative high quantum yield (the number of emitted photons/number of absorbed photons) and high fluorescence emission intensity (Lidke and Arndt-Jovin, 2004). The color (emission wavelength) of a QD is dependent on the size of the crystal, allowing the production of QDs with many different colors. A semiconductor nanocrystal, e.g. CdSe, is covered with a protective shell (ZnS), followed by a polymer coating and finally a layer of biocompatible molecules such as streptavidin, which can be used for the covalent binding of the QDs to biotinylated antibodies (**Figures 3.4 and 4.3**). Quantum dot SPT (QD-SPT) has been used extensively to study neurotransmitter receptor mobility in synaptic regions (Bannai et al., 2006; Dahan et al., 2003; Ehlers et al., 2007; Ehrensperger et al., 2007; Renner et al., 2010).

The subpixel, nanometer resolution that is possible in SPT is achieved through the precise determination of the center point of the object, which is obtained by fitting a two-dimensional gaussian surface to the intensity profile of the object. If the signal-to-noise ratio is high enough, only a few pixels are necessary to ensure a proper fit and a precise localization of the particle (**Figure 3.2**).

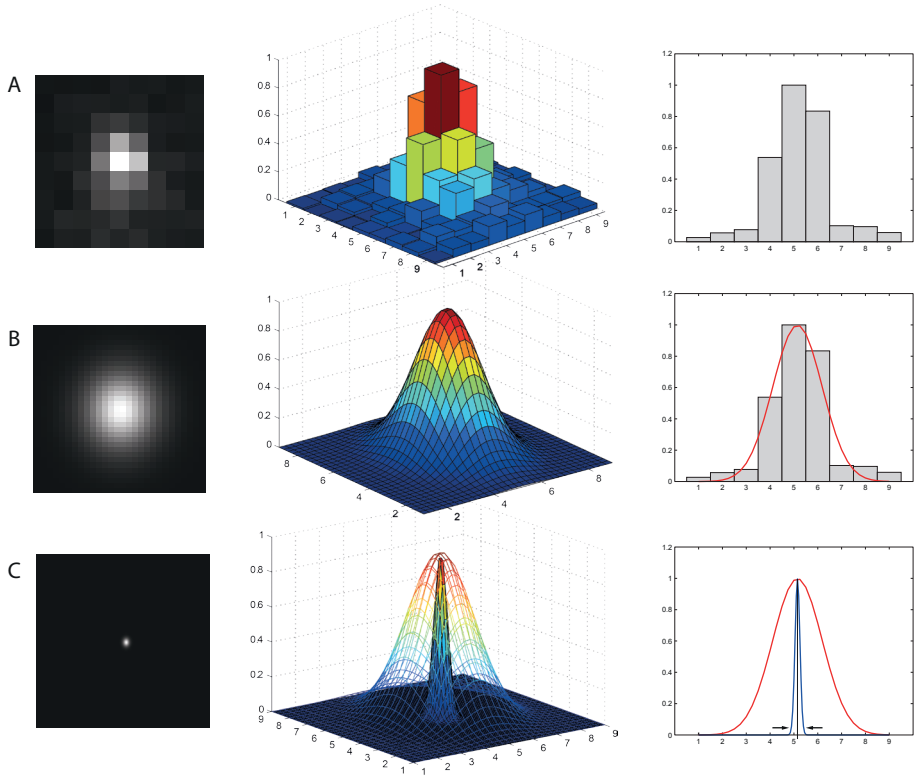

Figure 3.2: Subpixel resolution in SPT obtained using Gaussian fitting. A) Original image of a QD. B) Gaussian surface fit of the image in A. C) Center point of the QD. The accuracy of the method depends on the calculated error in the fitting step, and the stability of the system, which can be measured using an immobilized QD.

The localized particles are followed over time, and individual points belonging to the same particle are connected into trajectories. Mean square displacement, MSD, plots can then be calculated from the trajectories (Saxton and Jacobson, 1997). For free Brownian diffusion the initial slope of the MSD curve is proportional to the diffusion coefficient of the particle. An example of SPT performed on QD labeled Na,K-ATPase is shown in figure 3.3.

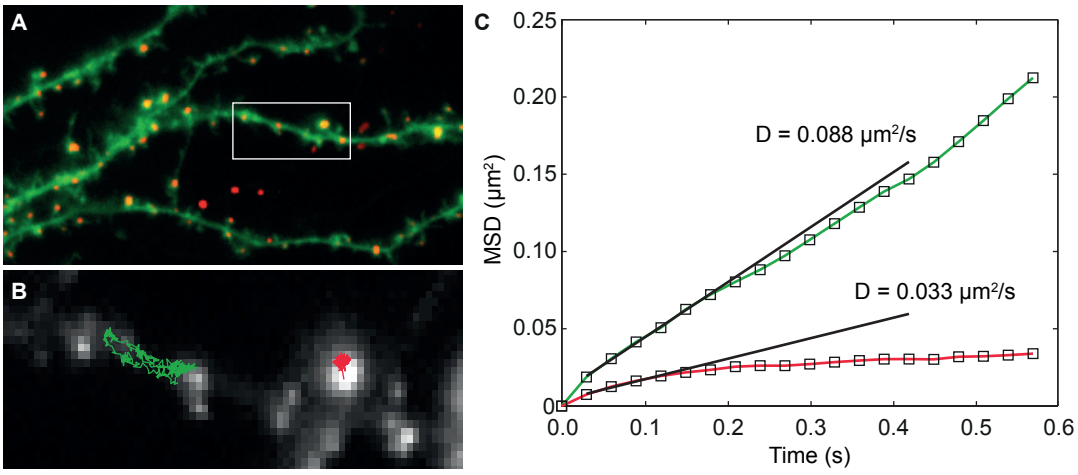

Figure 3.3: Example of QD recording and MSD plots. A) Hippocampal neuron expressing SEP-NKA-α3 and PSD-95-mCherry. B) Trajectories showing free diffusion (green) and confinement (red). C) MSD plots calculated from the two trajectories in B. The green and red plots describe typical free diffusion and confinement, respectively. Initial diffusion (D) is calculated from the linear slope (slope = 4Dt) of the first four (non-zero) points.

3.3.3 FRAP versus SPT

In FRAP experiments, what is measured is the flow of a population of unbleached (fluorescent) molecules into the bleached region, i.e. how much and how fast a certain region will be populated by the fluorescent molecule in question. Factors that impact the rate and extent of the diffusion into the bleached region include how mobile the fluorescent particles outside the bleached region are and how crowded the bleached region already is, i.e. how much space that is available for new molecules to occupy. The measured diffusion during steady-state conditions is the equilibrium of the diffusion of fluorescent particles into the bleached region and the movement of non-fluorescent particles out of the region, providing space in the bleached region. If the bleached region is crowded (e.g. the postsynaptic density at the head of a spine) the fluorescent particles will inhabit the region more slowly than if the region is less crowded (e.g. stretches of dendrite without postsynaptic terminals) resulting in an apparent slow diffusion even if the studied particles are mobile and freely diffusing. It is also possible that the bleached region is not crowded but the fluorescent molecules are bound and immobile which also will result in a slow apparent diffusion into the bleached region.

Single particle tracking, SPT, experiments on the other hand follow by design only the movements of a single particle. The caveat of SPT experiments is the randomness in the selection of the tracked molecules. In order to study discrete particles, with as little inter-particle trajectory intersection as possible, it is necessary to keep the number of labeled particles to a minimum. Not all of the identified molecules are located in regions that are of interest for the study in question. In order to achieve statistically significant data it is necessary to record the trajectories of many particles, excluding those that do not fit certain criteria. The benefits of SPT are the very high spatial and temporal resolution (< 15nm and <50 ms, respectively), not possible with FRAP.

FRAP and SPT are not interchangeable techniques used to answer the same questions, rather they are complementary techniques that can be used together in order to illuminate different properties of the mobility and diffusion of the studied particles. With FRAP, for example, it is possible to study the overall diffusion characteristic of a population of particles in a single recording. SPT, on the other hand, can be used to obtain parameters such as dwell time (the duration particles stay in a specific region), confinement and initial diffusion (Bannai et al., 2006). One must be careful when interpreting results from FRAP and SPT recordings.

Additional issues attributable to both techniques are the manner in how the particles in question are labeled. In FRAP experiments it is most common to attach a fluorescent protein to the studied protein. Because of this, it is important to ascertain that the function of the protein is not altered by the modification. It is equally important to evaluate the means of introducing the fused protein into the system, e.g. the cell, in order to minimize perturbations of the system caused by overexpression. In SPT recordings the particle labeling is usually achieved through quantum dot conjugated antibody labeling of the proteins. This requires highly specific primary antibodies and proper blocking of the sample to minimize unspecific background binding of secondary antibodies with quantum dots. One issue of this approach is the inherent large size of the resulting complex: the protein epitope is bound to a primary-secondary antibody complex, which in turn is attached to the outer biocompatible layer of a quantum dot. The size of this complex can be in the range of 20-40 nm (Lidke and Arndt-Jovin, 2004), in comparison a single GFP is approximately 5 nm (**Figure 3.4**). This issue might not affect the specific question (Dahan et al., 2003), but it should not be ignored when evaluating the results (Groc et al., 2004). There are other techniques that can be used for SPT, both modifying the size of the complex by using fewer and smaller intermediates or using other types of fluorescent probes than QDs, such as organic dyes (Groc et al., 2007) and hopefully there will be even more options available in the future.

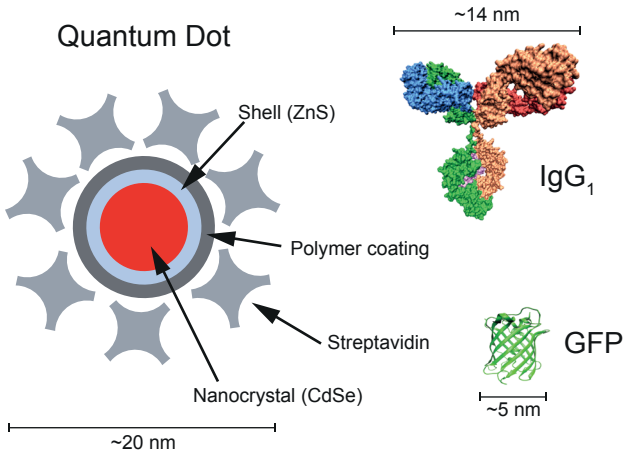

Figure 3.4: Size comparison between a QD, GFP and IgG antibody.

3.3.4 Isolated Na,K-ATPase currents, I_p

Na,K-ATPase pumps three sodium ions out of the cell in exchange for two potassium ions at the expense of one molecule of ATP. The net charge transfer per pump cycle will be one positive ion out of the cell, resulting in a hyperpolarization of the cell.

In order to directly measure the electrogenic current that is generated by the Na,K-ATPase (I_p) in intact neurons we used an electrophysiological protocol where the goal was to minimize all currents that are not due to the Na,K-ATPase (**Figure 3.5**). First we voltage-clamp the cell at 0 mV, which will inactivate the voltage-gated sodium channels (VGSC). The sodium concentration inside the pipette is 95 mM, which will decrease the driving force for sodium through VGSCs while ensuring maximal pump activity (V_{max}) of the Na,K-ATPase. The voltage-gated potassium channels (VGPC) are blocked by extracellular cesium and barium as well as intracellular tetraethylammonium (TEA) introduced via the pipette. Voltage gated calcium channels (VGCC) are blocked by extracellular cadmium and nickel as well as removal of calcium from the intracellular and extracellular solution, which also inhibits the sodium/calcium exchanger (NCX).

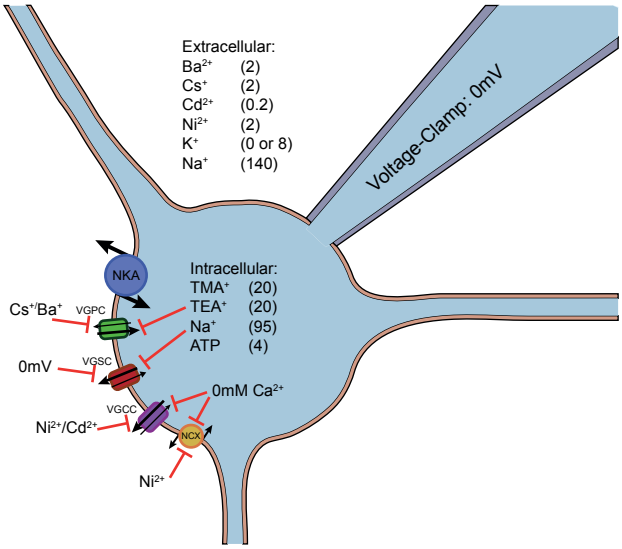

Figure 3.5: Protocol for isolation of the Na,K-ATPase current.

Pump turnover is dependent on the extracellular concentration of potassium and the intracellular concentration of sodium and ATP. Since the intracellular concentration of sodium and ATP were set to saturating levels we could turn the pump on and off by changing the extracellular concentration of potassium.

To measure the I_p we initially set extracellular potassium concentration to 0 mM, which will completely block pump turnover. We then increased the extracellular potassium concentration to 8 mM during a fixed timeframe (K^+-pulse), which will maximally activate the pump (**Figure 3.6**). After the first K^+-

pule we apply the treatment to the cells, e.g. ouabain or vehicle, and then perform a second K^+-pulse. The I_p is measured either as maximal amplitude or as the average current over the entire pulse duration (**Figure 3.6**). The I_p ratio is calculated as the ratio of the second I_p to the first I_p (second K^+-pulse to the first K^+-pulse).

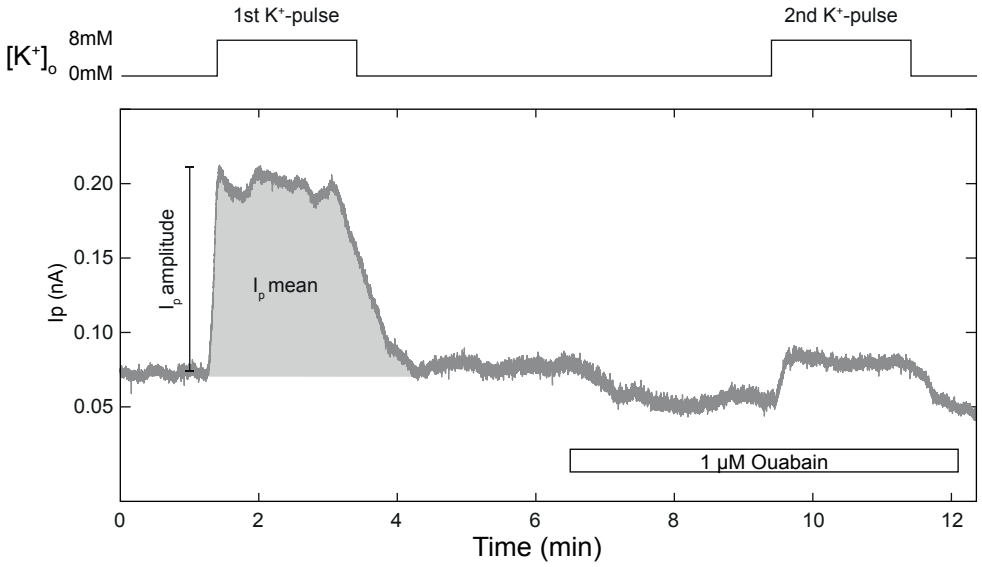

Figure 3.6: Example of I_p recording in hippocampal neuron.

This technique to measure the Na,K-ATPase specific current is very sensitive and has the additional benefit that the recording is cell-specific. Using this technique, it is thus possible to measure the I_p in different types of cells within a certain preparation, which is important in the process of elucidating the functional role of the α subunits in the brain. However, the method measures the current generated in the entire cell, with a possible bias toward the soma and proximal dendrites. The use of sodium-sensitive dyes is a good complement to this technique due to the possibility of recording changes in sodium levels anywhere in the neuron, such as in the distal dendrites, as we did in paper IV.

In conclusion, the importance of combining different techniques in order to overcome the shortcomings of each individual method cannot be overstated.

4 RESULTS AND DISCUSSION

4.1 Intracellular dynamics of Calcyon

The accessory protein calcyon was found in a study searching for potential D_1 c-terminal interacting proteins (Lezcano et al., 2000).
Our study on calcyon was initially based on the reported finding that calcyon by directly binding to D1R modifies the downstream effectors of D_1 signaling. However, we were not able to reproduce those findings. As it were, it was later explained that the initial findings were not correct (Lezcano et al., 2006).

During our initial experiments expressing recombinant calcyon fused to GFP in cell lines and neurons we observed that calcyon is localized in intracellular vesicles and that it is highly mobile, with a clear bi-directional transport pattern in dendrites of neurons. We set out to further describe the intracellular dynamics of calcyon irrespective of a possible connection with the dopamine receptors.

We show that calcyon is expressed in Neuronal Nuclei (NeuN, Mullen et al., 1992) - positive cells in the rat hippocampus, while no calcyon specific signal could be found in cell bodies that stained positive for Glial Fibrillary Acidic Protein, (GFAP, Eng et al., 2000). This finding indicates that calcyon is a neuron-specific protein and confirms what had previously been reported (Zelenin et al., 2002).

We then constructed a recombinant version of calcyon fused to the fluorescent protein Venus, which is a yellow-shifted mutated form of GFP (Nagai et al., 2002). We expressed this construct in HEK293 cells SH-SY5Y cells and both primary cultures of hippocampus and organotypic cultures of striatum. We observed highly mobile vesicle-like structures throughout the cytoplasm of HEK293 cells and in the soma and dendrites of neurons. The mobility of the vesicles decreased with lower temperature and was sensitive to treatment with nocodazole, suggesting microtubule-dependent transport (De Brabander et al., 1976). Furthermore we observed that many of the vesicle-like structures were located directly beneath the plasma membrane. This could be a) vesicles containing cargo destined for the plasma membrane that are about to fuse with the membrane (exocytosis); b) vesicles that have been endocytosed and are awaiting transport to lysosomes or endosomes; or c) a combination of both. In order to try to answer this question we treated the cells with fluorescent

wheat germ agglutinin (WGA) which is a compound that binds to surface proteins and remains attached during endocytosis and subsequent transport to the Golgi apparatus (Vetterlein et al., 2002). WGA only follows the endocytic pathway and does not leave the Golgi apparatus. After 45 minute treatment with WGA at 37 °C we observed WGA signal in a majority of the calcyon positive vesicles. Some vesicles did not contain any WGA. This suggests that calcyon vesicles are transported to the membrane where they fuse, followed by endocytosis and transport to intracellular organelles. It is still not clear what role calcyon has in this process, it has been suggested that calcyon is involved in CME where it is supposed to assist in the formation of clathrin-coated vesicles (Xiao et al., 2006).

We hypothesize that calcyon vesicles are stored beneath the plasma membrane as a ready-releasable pool of vesicles that can fuse with the membrane in response to stimulation. The nature and identity of the calcyon containing vesicles need to be further investigated. It is possible that calcyon is located in certain types of vesicles or that it is indiscriminately transported with different types of vesicles. It is also possible that the only role of calcyon is to be transported to the plasma membrane where it will assist in the formation of vesicles following the endocytic pathway. In order to answer these questions additional studies needs to be performed.

D1 Receptors are known to be regulated through the endocytic pathway; D1 activation signals a dynamin-dependent internalization of the receptor via clathrin-coated vesicles (Vickery and von Zastrow, 1999). Since calcyon is involved in CME, an interaction between calcyon and D1R, although not direct, is therefore still possible.

4.2 Functional differences between D_1 and D_5

Dopamine is an important modulatory neurotransmitter involved in a variety of cognitive and motoric functions. In this study we wanted to explore the functional difference between the two known isoforms of the D_1-like family: D_1 and D_5. Due to similar agonist/antagonist binding properties between D_1 and D_5 it has been difficult to investigate and discriminate the individual roles of the two isoforms.

Previous studies have shown that D_1 activation modulate NMDA receptor function via PKA dependent phosphorylation of the NMDA receptor, potentiating the NMDA receptor response to glutamate (Cepeda et al., 1993;

Flores-Hernandez et al., 2002) or via a direct protein-protein interaction between D_1 and NMDA-R that attenuates NMDA-R function (Scott et al., 2006) with a possible positive feedback mechanism where NMDA-R activation increase the expression of D_1 in the plasma membrane (Scott et al., 2002). These studies suggest a strong link between the D_1 and NMDA-R. However, the question remained whether this is true also for the other D_1-like receptor, the D_5. In order to further investigate this, we developed fluorescently tagged versions of D_1 and D_5; D_1-Venus and D_5-Venus. We expressed the constructs in primary and organotypic cultures of striatum and used confocal microscopy to study the interaction between D_5 and NMDA-R. FRAP recordings provided evidence that D_5, like D_1, move via lateral diffusion in the dendritic membrane. We used FRAP recordings to study whether NMDA-R activation would cause a decrease in the diffusion of D_5 receptors, which was previously shown to be the case for D_1. However, we found no effect of NMDA activation on D_5 mobility.

Furthermore, we also performed GST-pulldown experiments of the C-terminal parts of D_1 and D_5, the part of the two receptors with the least homology, with PSD-95. The PSD-95 is an integral protein in the postsynaptic density in excitatory synapses and has been implicated in the interaction between D_1 and NMDA-R (Gu et al., 2007). We found an interaction with the c-terminal tail of D_1 and PSD-95, but not between the c-terminal tail of D_5 and PSD-95.

A previous study described a direct protein-protein interaction between D_5 and the $GABA_A$ receptor (Liu et al., 2000). $GABA_A$ is a ligand-gated ion-channel that is one of the receptors for the major inhibitory neurotransmitter GABA, and is found predominantly on inhibitory synapses. However, FRAP recordings showed no change in mobility of D_5 when we treated the cells with GABA. These results do not completely exclude a connection between D_5 and NMDA-R or $GABA_A$, but a mobility-limiting interaction is less likely. Stimulation of these two receptors could still affect D_5 through other pathways, resulting in different downstream effects that do not lead to a changed mobility of D_5.

We also wanted to elucidate whether the subcellular localization of the two isoforms in neurons is dissimilar. Due to the poor supply of high quality antibodies that distinguish between D_1 and D_5 we decided to work with organotypical cultures of striatum expressing either D_1-Venus or D_5-Venus.We analyzed the expression of D_1-Venus and D_5-Venus in separate cultures by categorizing the fluorescence-positive spines. We found that D_1 and D_5 had somewhat differentiated expression patterns. D_1 was predominately expressed

in mushroom-like spines while D_5 was more prominent in stubby, thin and filopodia-like spines.
These findings indicate that D_1 and D_5 have different roles in the modulation of glutamatergic signaling.

Activation of the D_1-like receptors has been shown to potentiate excitatory transmission via phosphorylation of AMPA receptors (Greengard et al., 1999; Greengard et al., 1991), the findings of our study suggest a role for D_1 but not D_5 in this process. The development of isoform-specific pharmacological compounds is therefore an attractive pathway to more efficient treatment of a range of neurological disorders.

4.3 A non-canonical postsynaptic transport route for a GPCR

The lateral mobility and trafficking of excitatory neurotransmitter receptors have been the focus of several studies during the past decade (Borgdorff and Choquet, 2002; Ehlers et al., 2007; Heine et al., 2008; Makino and Malinow, 2009; Park et al., 2004; Sharma et al., 2006; Tardin et al., 2003). The spatial regulation of modulatory neurotransmitter receptors, or GPCRs, is much less studied.

During our previous projects we noticed that most of the GPCRs we studied, when expressed in neurons, displayed an even distribution in the somatodendritic plasma membrane. We decided to find out if a general pathway exists for GPCR trafficking in neurons. Two possible transport pathways for receptors to the active site are shown in figure 4.1.

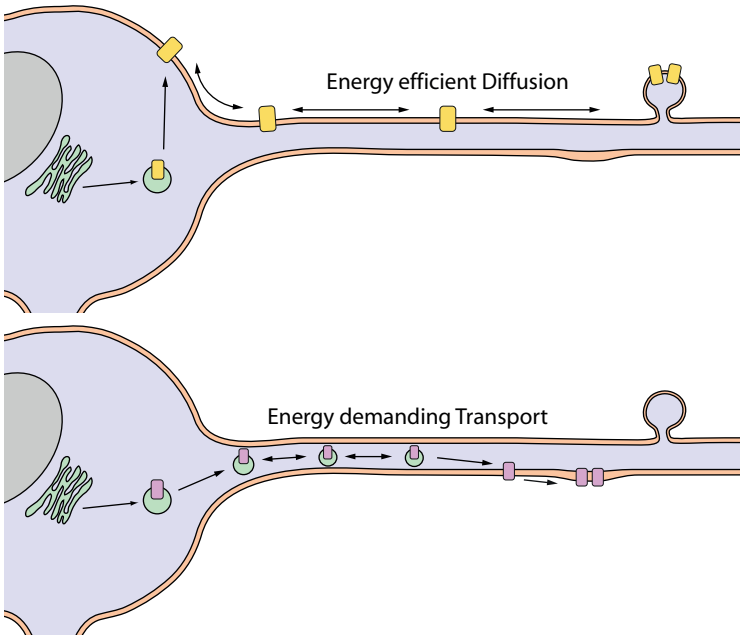

Figure 4.1: Example of two routes of transport. Top: Energy efficient transport common for many GPCRs. Bottom: Active transport of receptor-containing dendritic vesicles to active site.

We selected seven GPCRs that are involved in mood regulation and implicated in neurological disorders such as schizophrenia or depression. We selected three dopamine receptors (D_1, D_2 and D_5), three serotonin receptors (5-HT_{1A}, 5-HT_{1B} and 5-HT_4), and finally the metabotropic glutamate type 5 receptor (mGluR5). In order to monitor the intracellular distribution and mobility of the different receptors, we tagged the receptors with fluorescent proteins (FP) and expressed them in primary hippocampal neurons. We assessed the transport of fluorescently labeled receptors by FRAP, a method that is well suited for the documentation of diffusion. We used kymographs to visualize the recovery. Membrane diffusion was apparent in all kymographs except in the one for 5-HT_{1B}. In the 5-HT_{1B} kymograph, bright slanted lines appeared, indicating directed transport of clusters into the bleached region.
FRAP recordings on the soma-dendrite junction revealed the transport of 5-HT_{1B} clusters out of the soma toward the dendrites. We used PA-GFP-tagged 5-HT_{1B} to further visualize this transport. The soma of cell transfected with PA-GFP-5-HT_{1B} was illuminated with a 405 nm laser beam and the soma-proximal dendrite region was recorded. Clusters were visible in the dendrites after activation of the soma, indicating the soma as the source of the vesicles.

One major drawback when expressing an exogenous variant of a protein in cells is the risk of over-expression artifacts. To rule out such artifacts in this study we performed a series of control experiments. The expression pattern of the transfected FP-5-HT_{1B} was similar to the endogenous 5-HT_{1B}. Exchanging the FP

to a smaller hemagglutinin (HA) tag did not change the expression. The level of expression of FP-5-HT_{1B} did not alter the pattern of expression either. In conclusion, we got no indication that the expression pattern of FP-5-HT_{1B} was due to over-expression artifacts.

Next we wanted to map the transport pathway, what kind of vesicle is transporting the 5-HT_{1B}? We co-expressed FP-5-HT_{1B} with a set of vesicular markers. In our assay we chose a set of Rab proteins as well as clathrin and caveolin, all known to be involved in different steps of vesicular transport (Pearse, 1976; Rothberg et al., 1992; Stenmark and Olkkonen, 2001). 5-HT_{1B} clusters were found to be highly associated with Rab8a and caveolin. Rab8a is involved in the transport of nascent proteins from the Golgi apparatus to the plasma membrane. The caveolin association is likely a counterbalance to the secretory pathway as caveolin is involved in endocytosis.

To study the recruitment of 5-HT_{1B} receptors to the plasma membrane we used the pH sensitive version of GFP, super ecliptic pHluorin (SEP). The SEP was introduced in an extracellular loop. This way the SEP would be eclipsed as long as the receptor was located in a vesicle, due to the low pH. As soon as the vesicle docked with the membrane the vesicular lumen would neutralize and the SEP would become fluorescent. This was seen as a rapid increase in fluorescence in the membrane, followed by a slower radial diffusion from the docking point. The vesicle recruitment was found to be activity-dependent as depolarization of the membrane resulted in an increased frequency of events. We mapped the recruitment sites and found that they were not evenly distributed on the membrane but rather preferred certain regions. When mapped against the inhibitory synapse marker gephyrin, we observed that the receptors were more likely to be recruited close to an inhibitory synapse.

Finally we studied the trafficking of receptors to the synapse. We labeled the 5-HT_{1B} with quantum dots and used single particle tracking to trace individual receptors. The receptors were found to be moving by lateral diffusion and were confined in inhibitory (gephyrin positive) and excitatory (PSD-95 positive) synapses.

In this study we describe a novel transport route for a GPCR, from the soma out to the site of action, the inhibitory synapse. We propose that this special type of transport, serves as an additional mode of regulation, which enables fine-tuning of serotonergic signaling.

4.4 Essential postsynaptic role of the Na,K-ATPase α3

The Na,K-ATPase has a fundamental role in setting the ion-gradients powering a majority of biological processes and in maintaining the membrane potential that is critical for neuronal signaling. The different Na,K-ATPase α subunit isoforms are probably a result of evolution where properties crucial for a certain cell type, e.g. a neuron, have been optimized over time.

In this study we wanted to elucidate the role of the two α-subunit isoforms, α1 and α3 in neurons. Why do neurons express the α3, is it necessary for the type of processes that occur during neuronal signaling? In order to try to answer this question we utilized the fact that, in rats, the two isoforms differ a lot in terms of sensitivity to ouabain, a steroid hormone that is a specific ligand and inhibitor to Na,K-ATPase (Cornelius and Mahmmoud, 2009; Lingrel, 2010; Schatzmann, 1953). The α3 isoform is more than a hundred times more sensitive to ouabain than α1. Using ouabain at a dose of 1 μM we could completely inhibit the α3, while the α1 is inhibited to less than 10% (Sweadner, 1985; Urayama and Sweadner, 1988).

In our study we used primarily hippocampal neurons in primary culture. Using immunocytochemistry we show that all neurons contain both α1 and α3, but at different levels. Even though inherent differences in antibody affinity make any proper quantification based on immunoreactive signal difficult, it was apparent that α3 was expressed to a higher degree in the neurons. It was also clear that the level of α1 was higher in astrocytes than in neurons.

Next we measured the effect of α3 inhibition on the ability of the Na,K-ATPase to maintain the membrane potential and basal level of intracellular sodium. If the pump is blocked, the sodium concentration will increase in the cell, which subsequently will result in depolarization of the membrane potential. A small change in intracellular sodium will result in a larger change in membrane potential. Using the current-clamp protocol while applying 1 μM ouabain we recorded a depolarization of the cell, which initially triggered increased firing and finally led to loss of activity in the cell. When we instead monitored the intracellular sodium concentration, using the sodium-sensitive indicator Asante Natrium Green 1 (ANG), we observed only a minor increase in intracellular sodium when treating the cells with 1 μM ouabain. A much larger increase was, not surprisingly, observed when the cells were treated with 1 mM ouabain,

which should completely inhibit both isoforms. The sodium influx is related to the activity of the culture, and it is likely that blocking the pump in an active cell, i.e. a cell that receives a lot of excitatory input, will result in a more rapid increase in intracellular sodium than in a cell that does not receive any input. The activity was not directly measured in the neurons used for the measurement of intracellular sodium. However, the same cultures were used for current-clamp recordings and they displayed high spontaneous activity. The minor increase in intracellular sodium together with the relatively strong effect on membrane potential suggest that α1 activity is enough to maintain basal sodium levels but is not sufficient in sustaining the membrane potential.

When we had verified that the neurons expressed both isoforms we used two protocols to study the function of α3. The first protocol was an electrophysiological method that was designed to isolate the electrogenic Na,K-ATPase current (I_p, explained in the methods section). Using this method, we found that when the cells were treated with 1 μM ouabain, the I_p was almost completely abolished, indicating that α3 is the dominant isoform in these neurons. In the second protocol we triggered an influx of sodium by transiently blocking pump activity using potassium free extracellular solution and monitored the recovery of sodium to baseline levels when potassium was reintroduced to the extracellular buffer. The influx of sodium was comparable to levels reached after suprathreshold stimulation (Rose and Konnerth, 2001). Using this protocol we found that α3 inhibition severely decreased the recovery rate, indicating an essential role for α3 in handling high levels of intracellular sodium. In fact, the intracellular sodium level never returned to base line levels in cells treated with 1 μM ouabain, the sodium influx resulted in some irreversible loss of function in these cells.
This study suggests an essential role for α3 in handling the transient increases in intracellular sodium associated with high neuronal activity. We show that the α1 is not sufficient in handling these processes. These findings may help explain the severe disorders that are a result of mutations in human Na,K-ATPase α3.

4.5 Postsynaptic mobility of Na,K-ATPase α3 – ongoing project

The findings in paper IV prompted us to further investigate the postsynaptic role of α3 and to study whether the mobility of α3 is regulated in an activity-dependent manner.
The function of a postsynaptic protein is highly dependent on sub-cellular localization. It is now clear that most membrane proteins move via lateral

diffusion and that regulation of this mobility is important for the overall protein function. In order to study the spatial regulation of the Na,K-ATPase α-subunits in dendritic membranes, we constructed fluorescent protein-tagged variants of the α1 and α3 subunits. One set of constructs was made by adding GFP or RFP to the N-terminal of the subunits; GFP-NKA-α1 and RFP-NKA-α3. These constructs were used to study the overall localization and mobility of the α-subunits. We performed FRAP experiments on spines of primary hippocampal neurons expressing GFP-NKA-α1 and RFP-NKA-α3 constructs. We found similar diffusion characteristics between the α1 and α3 subunits, with a high mobile pool and a relatively fast diffusion profile (**Figure 4.2**).

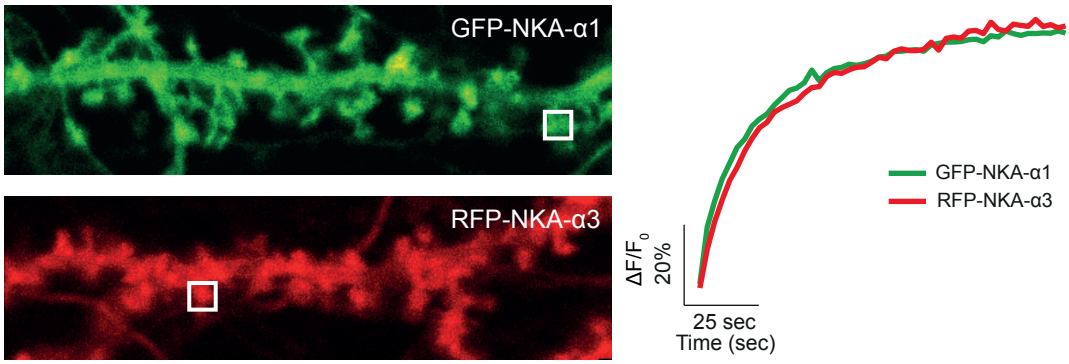

Figure 4.2: FRAP recordings of GFP-NKA-α1 and RFP-NKA-α3 showing a rapid recovery and a high mobile pool.

In order to visualize the membrane specific fluorescence of the Na,K-ATPase, we designed another construct by inserting a pH-sensitive variant of GFP, Super Ecliptic pHluorin (SEP) (Miesenbock et al., 1998; Sankaranarayanan et al., 2000), into the second extracellular loop of the Na,K-ATPase α3 subunit, SEP-NKA-α3 (**Figure 4.3 A**). Since both the N-terminus and the C-terminus of the α subunit are located intracellularly, this approach made it possible to express an α subunit with an extracellular pH-sensitive GFP, that in addition of enabling membrane specific fluorescence, serves as a highly specific epitope useful for antibody labeling in QD-SPT experiments (**Figure 4.3 B**).

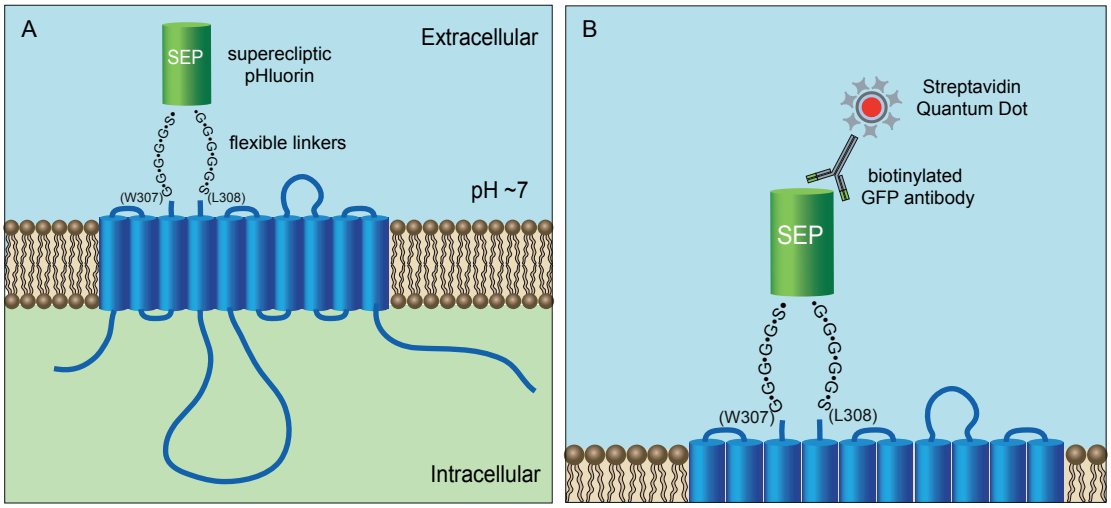

Figure 4.3: The use of an extracellularly located SEP for FRAP and QD labeling.

We transfected hippocampal neurons with the SEP-NKA-α3 and compared the diffusion between spines and equally sized regions of dendrites using FRAP. We noticed that the diffusion in the spine was slower than in the dendrites (**Figure 4.4**). The dendritic plasma membrane resembles a two-dimensional plane, and the spine, which can be represented by a sphere connected to the dendrite via a small neck, will have different diffusion profiles. The observed decreased diffusion is therefore likely due to a combination of geometrical differences and confinement of NKA-α3 in the head of the spine.

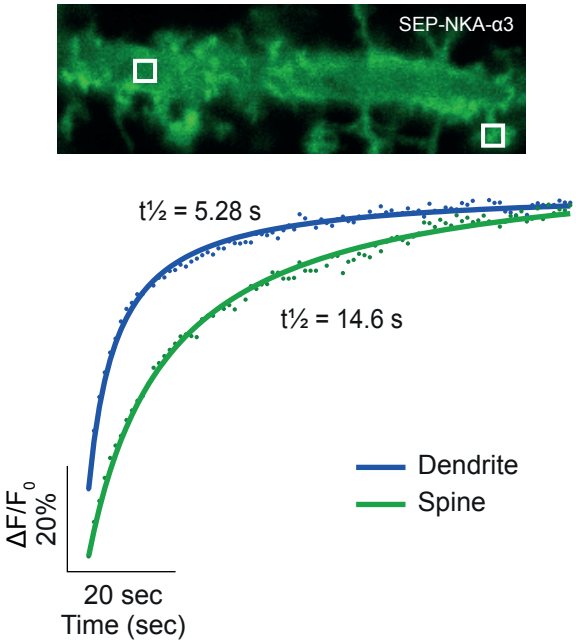

Figure 4.4: Comparison between diffusion of SEP-NKA-α3 in spines and dendrites.

The membrane depolarization resulting from excitatory synaptic activity is due to influx of Na^+ ions through ionotropic glutamate receptors into the spine

(**Figure 4.5**). This increase in intracellular Na^+ must be counterbalanced by active extrusion via the Na,K-ATPase. Several studies in recent years have described the activity-dependent regulation of glutamate receptor mobility in the synaptic space (Borgdorff and Choquet, 2002; Ehlers et al., 2007; Heine et al., 2008; Sharma et al., 2006; Tardin et al., 2003). Based on these findings we hypothesized that the mobility of the Na,K-ATPase is regulated by synaptic activity.

To test whether an increased $[Na^+]_i$ would alter the mobility of the Na,K-ATPase we treated the cells with AMPA. In order to show the effect of AMPA on the neurons, and in particular the $[Na^+]_i$ in spines, we designed an experiment where we loaded a neuron with a Na^+-sensitive dye, CoroNa-Green, via a patch pipette. By applying AMPA via a second pipette we could monitor both the resulting depolarization of the cell and the increase in $[Na^+]_i$ in spines and dendrites (**Fig. 4.5 and Fig. 4.6**).

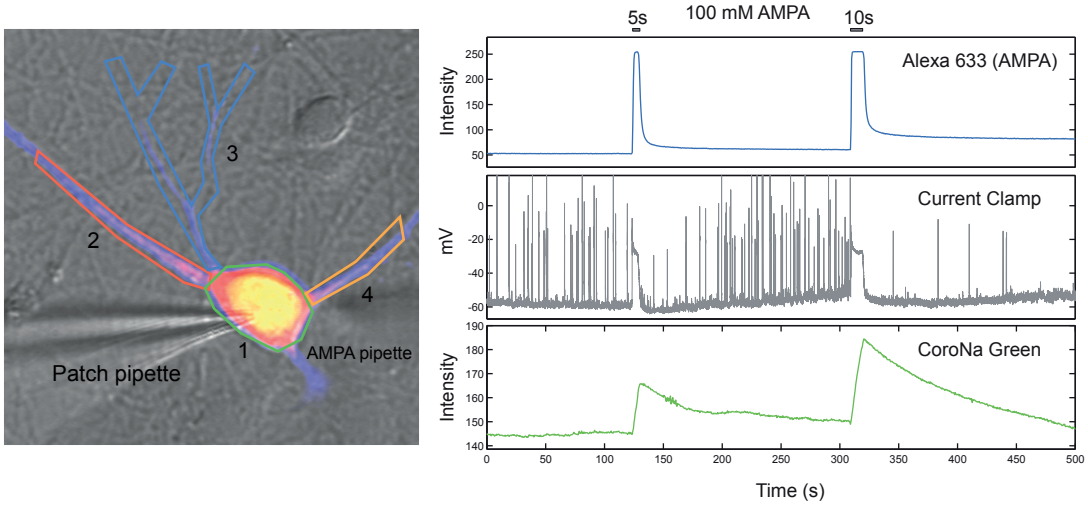

Figure 4.5: CoroNa loaded cells and pressure application of AMPA.

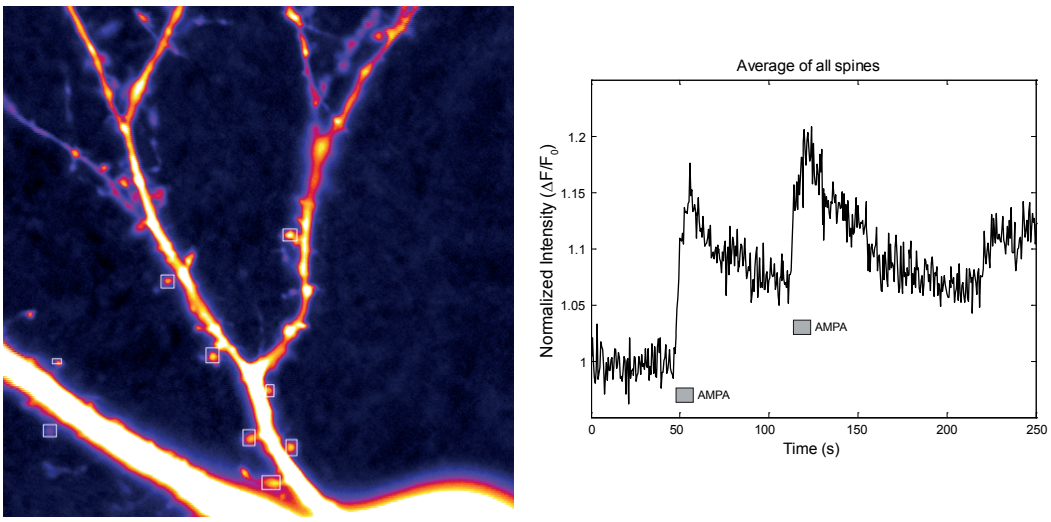

Figure 4.6: $[Na^+]_i$ increase due to application of AMPA measured in spines using Na+ sensitive dye CoroNa-Green.

Next we performed FRAP experiments on neurons transfected with SEP-NKA-α3 and treated with 5µM AMPA for 30 seconds. We noticed an increased mobility of α3 in spines of neurons treated with AMPA compared to control (**Fig. 4.7**).

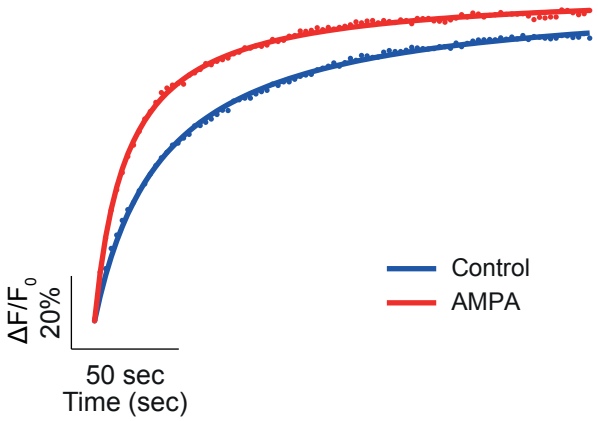

Figure 4.7: AMPA treatment increase diffusion of α3 in spines.

In order to study in more detail how α3 is moving in the synapse we used single particle tracking. We took advantage of the fact that antibodies raised against GFP are highly specific, due in part to the special barrel-shape structure of the GFP (Ormo et al., 1996; Yang et al., 1996) and the fact it is not a mammalian protein. Placing the SEP in the second extracellular loop of NKA α3 enabled us to bind quantum dots to the SEP-NKA-α3 with high specificity using biotinylated antibodies and streptavidin coated quantum dots (**Fig. 4.5.1B**). In order to label the excitatory synapses we co-expressed the neurons with PSD-95-mCherry (**Fig. 4.5.7**).

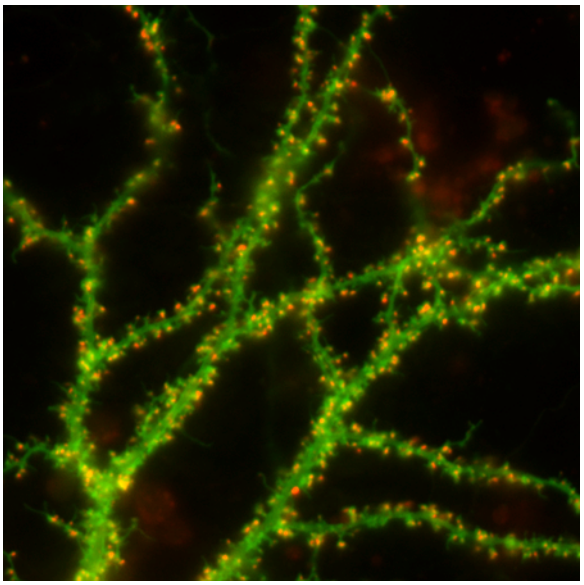

Figure 4.8: Dendrites of hippocampal neuron transfected with SEP-NKA-α3 (green) and PSD-95-mCherry (red).

In accordance with the FRAP data we found that the majority of α3 molecules in the dendritic membrane were highly mobile. The diffusion of α3 in the excitatory synapse was found to be slower compared to the diffusion in extrasynaptic regions. (**Fig. 4.9**).

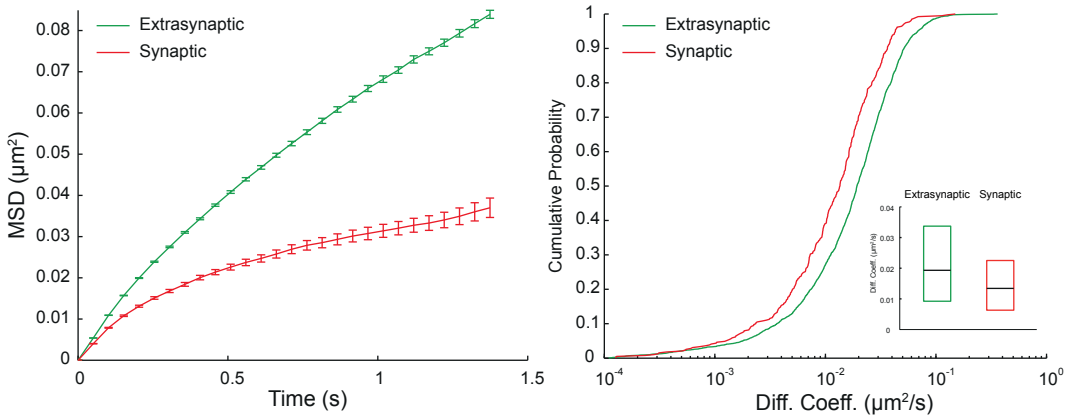

Figure 4.9: Mean Square Displacement (MSD) plot and Cumulative Probability plot of synaptic and extrasynaptic diffusion of NKA α3.

The mean square displacement (MSD) plots of molecules found inside the synaptic region show that the α3 is confined in the synapse.
When the cells were treated with AMPA we noticed that the mobility of the α3 molecules increased in both the extrasynaptic and the synaptic region (**Fig. 4.10**).

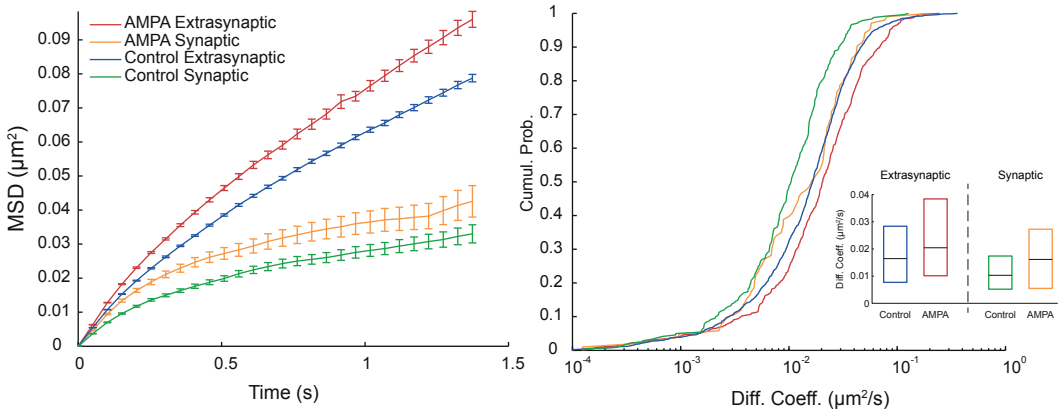

Figure 4.10: MSD plot and Cumulative Probability on α3 mobility after treatment with 5µM AMPA for 30 seconds.

In this study we search for a link between the neuron specific Na,K-ATPase α3 isoform, a protein essential for the generation of membrane ion gradients and for maintaining the membrane potential, and AMPA receptors, the principal excitatory receptor responsible for the depolarization of the membrane during synaptic activity.

Using FRAP and SPT, we demonstrate that AMPA receptor activation increases the mobility of α3 in the extrasynaptic and synaptic region. This AMPA-R-dependent regulation of α3 mobility will impact pump distribution and availability at local sites of sodium influx. Hypothetically, AMPA receptor activity might induce small conformational changes in the α subunit or it might trigger changes in protein interactions that alter the membrane mobility of the α3 isoform. Regulation of α3 mobility likely plays an important role in the maintenance of intracellular sodium in neurons.

5 CONCLUSIONS

Calcyon is a neuron-specific vesicular protein that follows the endocytic recycling pathway and is stored in ready-releasable pools beneath the plasma membrane. We propose a role for calcyon in the trafficking of proteins to and from the postsynaptic membrane.

The dopamine receptor D_1, but not D_5, interacts with NMDA receptors and PSD-95. We propose that D_1 is confined in the postsynaptic membrane upon NMDA stimulation and is responsible for the intra-neuronal interaction between the dopaminergic and glutamatergic systems, while D_5 appears not to be involved in this interaction.

The G-protein coupled receptor 5-HT_{1B} follows a non-canonical pathway for GPCR delivery where receptor-containing vesicles are transported inside the dendrites pending activity-dependent membrane recruitment. Membrane recruited 5-HT_{1B} receptors diffuse laterally and are confined at the active site, the inhibitory synapse. This specialized mode of transport is likely to serve as an additional layer of signal regulation of the serotonergic pathways.

The neuron-specific Na,K-ATPase α3 is the dominant isoform in hippocampal neurons and it is required to manage the influx of sodium in the postsynaptic region that is a consequence of excitatory synaptic activity. Na,K-ATPase α3 is highly mobile in the postsynaptic membrane. The mobility of the α3 is altered by excitatory glutamate receptor activation. This regulation of α3 mobility will impact pump distribution and availability at local sites of sodium influx, which is likely to impact synaptic activity due to changes in sodium homeostasis and membrane potential. These findings may help explain the severe neurological consequences of human α3 mutations.

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